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TRANSFORMING GROWTH FACTORS $\beta 1$ AND α IN CHRONIC LIVER DISEASE

Effects of Interferon Alfa Therapy

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Abstract Background. Cirrhosis is a diffuse process of hepatic fibrosis and regenerative nodule formation of unknown pathogenesis. Transforming growth factor (TGF) $\beta 1$ induces the production of extracellular matrix proteins by liver cells and has been implicated in the pathogenesis of hepatic fibrosis in laboratory animals. TGF α is a hepatocyte mitogen that participates in liver regeneration.

Methods. Using Northern blot analysis, we studied the expression of TGF $\beta 1$ messenger RNA (mRNA) in liver specimens from 42 patients with chronic hepatitis and cirrhosis and 12 subjects with either normal or fatty livers. The results were correlated with measurements of procollagen Type I mRNA in liver tissue, procollagen Type III peptide in serum, and the degree of histologic injury. We also investigated whether TGF α mRNA would be detectable in biopsy specimens of livers with proliferative activity.

Results. TGF $\beta 1$ mRNA expression correlated closely with the expression of procollagen Type I mRNA

($r = 0.94$) and serum procollagen Type III peptide ($r = 0.89$) and with the histologic activity index ($r = 0.73$). All patients with increased fibrogenic activity (serum procollagen Type III peptide level, $>11.9 \mu\text{g}$ per liter) had increased levels of TGF $\beta 1$ mRNA (2 to 14 times the levels in the control group or in patients with normal fibrogenic activity), and both TGF α and H3 histone (a marker of DNA synthesis) mRNAs were detectable in patients with regenerative nodules. Six of eight patients with hepatitis C treated with interferon alfa for one year had sustained clinical responses with normalization of serum procollagen Type III peptide and aminotransferase activity. All these patients had normal levels of TGF $\beta 1$ mRNA in liver specimens obtained at the end of the year.

Conclusions. TGF $\beta 1$ may have an important role in the pathogenesis of fibrosis in patients with chronic liver disease, and TGF α expression may be associated with liver regeneration in these patients. (N Engl J Med 1991; 324:933-40.)

FIBROSIS is an important component of advanced chronic inflammatory liver disease.¹⁻⁵ In cirrhosis, fibrosis is accompanied by cell necrosis and nodular regeneration.^{2,3} Hepatic fibrosis is a complex process that involves changes in the amounts of extracellular matrix components, activation of cells capable of producing matrix materials, cytokine release, and tissue remodeling.^{1,4-7}

In addition to being an inhibitor of DNA synthesis by hepatocytes,⁸⁻¹² transforming growth factor (TGF) $\beta 1$ is associated with hepatic fibrosis.¹³⁻¹⁵ TGF $\beta 1$ increases the production of extracellular matrix proteins and their receptors and inhibits the synthesis of matrix-degrading proteolytic enzymes.¹⁶⁻²⁰ In the liver, TGF $\beta 1$ induces collagen synthesis in lipocytes.²¹⁻²⁴

These cells, located in subendothelial spaces, are believed to become activated in hepatic fibrosis.^{1,4,6,23-27} Using an experimental model of schistosomiasis in mice, Czaja et al.¹³ found an increase in the content of TGF $\beta 1$ messenger RNA (mRNA) in the liver of infected animals, in parallel with an increase in Type I procollagen mRNA. Nakatsukasa et al.²⁴ also found a positive correlation between TGF $\beta 1$ mRNA and procollagen I, III, and IV mRNAs in the livers of rats with carbon tetrachloride-induced hepatic fibrosis. In view of these observations, we wondered whether the expression of TGF $\beta 1$ mRNA would correlate with various indicators of liver fibrosis and injury in humans. We therefore measured the steady-state levels of TGF $\beta 1$ mRNA in liver biopsy specimens from patients with chronic hepatitis and cirrhosis and correlated its expression with that of procollagen Type I mRNA in the same biopsy specimen and with serum levels of procollagen Type III peptide. To determine the extent of proliferative activity in the livers of patients with chronic hepatic disease, we measured the expression of H3 histone mRNA, generally considered a good marker of DNA synthesis.²⁸⁻³⁰ Since TGF α is a potent hepatocyte mitogen produced in vivo and in vitro by hepatocytes stimulated to proliferate,^{9,31}

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dehyde-3-phosphate dehydrogenase mRNA (2 hours of exposure) in the same biopsy sample.

Amino-Terminal Peptide of Procollagen Type III

Serum procollagen III amino-terminal peptide was measured with a commercial radioimmunoassay kit (RIA-gnost P-III-P; Behringwerke AG, Marburg, Germany). Normal values (mean \pm SD, 9.9 ± 1.0 μ g per liter) were established from serum measurements in 12 normal subjects (4 women and 8 men, ranging in age from 29 to 49 years; mean age, 39).

Viral Markers

Commercially available radioimmunoassay kits were used to detect hepatitis B surface and e antigens; antibodies to the surface, e, and core antigens; and anti-hepatitis delta antibodies (AUSRIA II, AUSAB, CORAB, HBe kit, and anti-Delta kit; Abbott Laboratories, North Chicago). Serum levels of hepatitis B virus DNA and DNA-polymerase activity were measured by dot hybridization and enzymatic reaction as previously reported.^{32,33} Antibodies against hepatitis C and HIV-1 were determined by enzyme-linked immunosorbent assay (Ortho, Raritan, N.J., and Abbott Laboratories, respectively).

Liver Biopsies

Biopsy specimens were graded for their degree of periportal, portal, and lobular inflammation and fibrosis according to the scoring system of Knodell et al.³⁴ Coded specimens were examined by a pathologist who had no knowledge of their source.

Statistical Analysis

The results are presented as means \pm SD. The results were evaluated by analysis of variance, polynomial regression, the Mann-Whitney test, the Wilcoxon signed-rank test, and Fisher's exact test.

RESULTS

TGF β 1 mRNA Expression and Fibrogenesis

We first determined whether the various indicators of fibrogenesis correlated with each other in the 42 patients with chronic hepatitis or cirrhosis. The expression of procollagen Type I mRNA in liver specimens on Northern blot analysis correlated directly with the serum levels of procollagen Type III amino-terminal peptide ($r = 0.91$, $P < 0.001$) (Fig. 1A). Furthermore, the serum levels of procollagen Type III peptide correlated significantly with the serum levels of ALT ($r = 0.84$, $P < 0.001$) (Fig. 1B). We then measured the expression of TGF β 1 mRNA and procollagen Type I mRNA in each specimen from the patients with chronic hepatic disease and the control group (Fig. 2). To eliminate variations that might be caused by technical procedures, the levels of TGF β 1 mRNA and procollagen Type I mRNA in each patient were calculated in relation to the level of glyceraldehyde-3-phosphate dehydrogenase in the same sample (Fig. 2). An analysis of the results in all patients revealed that the expression of TGF β 1 in the biopsy specimens correlated strongly with that of liver procollagen Type I mRNA ($r = 0.94$, $P < 0.001$; $n = 32$) and with the serum levels of procollagen Type III peptide ($r = 0.89$, $P < 0.001$) (Fig. 3). Liver concentrations of TGF β 1 mRNA and serum concentrations of procollagen Type III peptide were low in the control patients (mean procollagen Type III peptide concentration, 10.0 ± 0.3 μ g

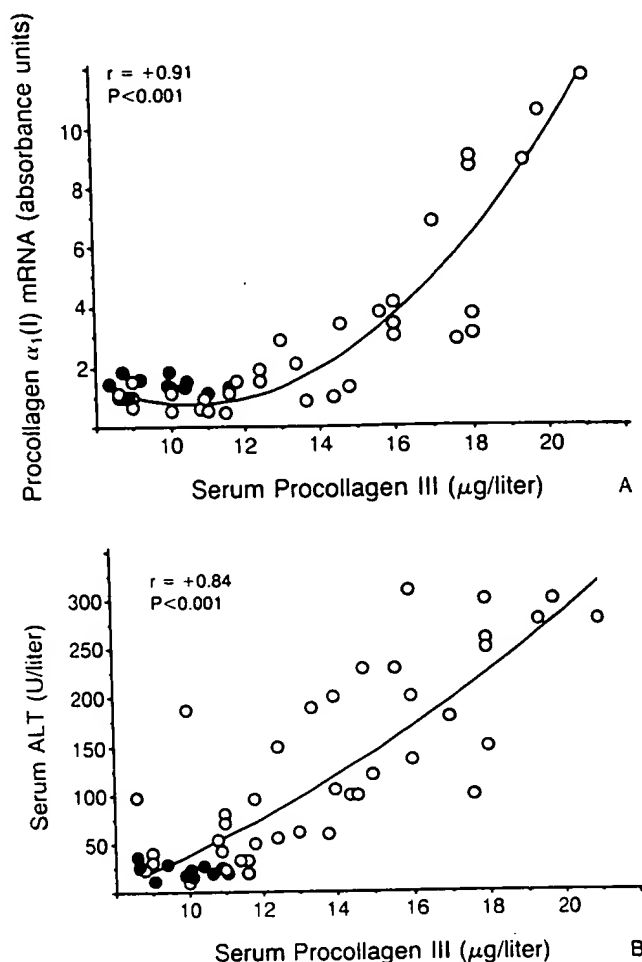


Figure 1. Serum Concentrations of Procollagen Type III Peptide in Relation to Hepatic Procollagen $\alpha_1(I)$ mRNA Expression (A) and Serum ALT Activity (B) in Patients with Chronic Liver Disease (O) and Control Patients (●).

In Panel A, the procollagen $\alpha_1(I)$ mRNA content in 34 patients and 12 control patients has been normalized to that of glyceraldehyde-3-phosphate dehydrogenase mRNA as described in Methods, and expressed in densitometric absorbance units. The mean (\pm SD) serum procollagen III values in the control group averaged 10.0 ± 0.3 μ g per liter (range, 8.6 to 11.6).

Panel B shows serum ALT activity in 40 patients with chronic liver disease and the 12 control patients. The mean (\pm SD) value in the control group was 25 ± 4 U per liter (range, 18 to 32).

per liter) but varied widely in the patients with chronic liver disease (Fig. 3).

To analyze in more detail the relation between TGF β 1 mRNA expression and fibrogenesis, the patients were divided into two groups according to the serum level of procollagen Type III peptide: those with high fibrogenic activity (serum procollagen Type III peptide > 11.9 μ g per liter, corresponding to 2 SD above the mean in the normal subjects) and those with normal fibrogenic activity. The patients with high fibrogenic activity had increased levels of TGF β 1 mRNA, ranging from 2 to 14 times above the amounts found in the control group, whereas the patients with normal fibrogenic activity had TGF β 1 mRNA levels

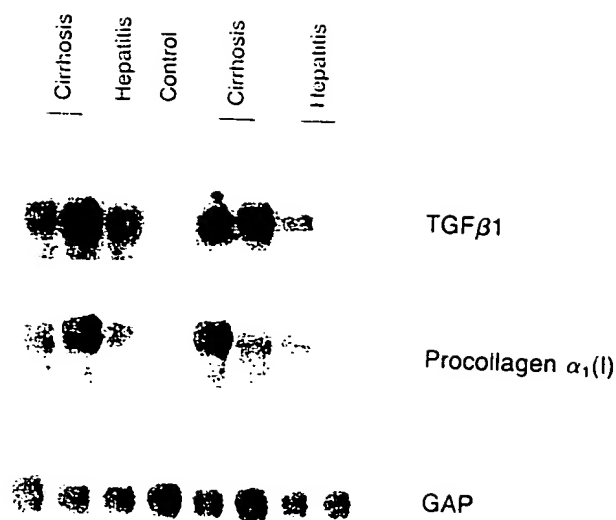


Figure 2. Representative Northern Blot Analysis of TGFβ1, Procollagen α₁(I), and Glyceraldehyde-3-Phosphate Dehydrogenase (GAP) mRNAs in Liver Specimens from Control Patients, Patients with Chronic Hepatitis, and Patients with Cirrhosis.

that did not differ from the level in the control group (Fig. 4). Although there was considerable variation, the highest levels of hepatic TGFβ1 mRNA were detected in patients with cirrhosis who had high fibrogenic activity.

TGFβ1 mRNA Expression and Tissue Injury

The histologic activity index described by Knodell et al.³⁹ can be used as a semiquantitative method to assess the degree of histologic injury in chronic liver disease. For each patient, the scores for the total index and some subindexes were compared with the levels of TGFβ1 mRNA in the same biopsy sample. TGFβ1 mRNA expression correlat-

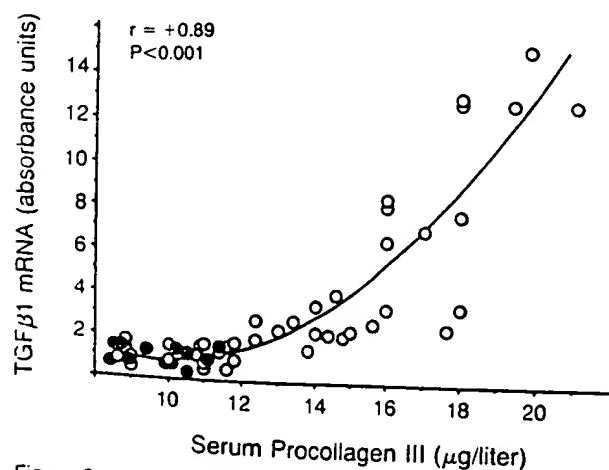


Figure 3. Hepatic TGFβ1 mRNA Expression in Relation to the Serum Concentration of Procollagen Type III Peptide in 42 Patients with Chronic Liver Disease (C) and 12 Control Patients (●). TGFβ1 mRNA content was normalized to that of glyceraldehyde-3-phosphate dehydrogenase mRNA and expressed in densitometric absorbance units.

ed directly with the total index ($r = 0.73$, $P < 0.001$) and some subindexes (piecemeal necrosis: $r = 0.67$, $P < 0.001$; fibrosis: $r = 0.69$, $P < 0.001$; lobular injury: $r = 0.54$, $P < 0.005$; and portal inflammation: $r = 0.53$, $P < 0.01$).

Expression of H3 Histone and TGFα mRNAs in Chronic Liver Disease

To assess the level of proliferative activity in the livers of the 42 patients and the control group, we examined the expression of H3 histone gene mRNA in

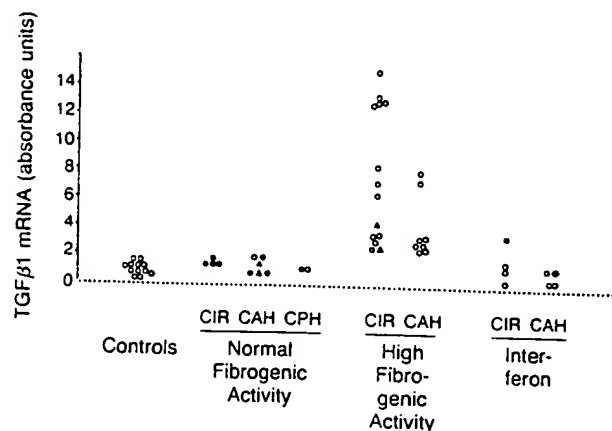


Figure 4. Hepatic TGFβ1 mRNA Expression in Control Patients and Patients with Chronic Liver Disease Untreated or Treated with Interferon.

The patients with chronic liver disease were divided into three groups: those with high fibrogenic activity (a serum procollagen Type III peptide concentration higher than 11.9 μg per liter, corresponding to the mean +2 SD in normal subjects), those with normal fibrogenic activity, and those with chronic hepatitis C virus infection and high fibrogenic activity who were treated with interferon for one year. For each patient, TGFβ1 mRNA content was normalized to that of glyceraldehyde-3-phosphate dehydrogenase mRNA, as described in Methods. CIR denotes cirrhosis, CAH chronic active hepatitis, and CPH chronic persistent hepatitis. The interferon-treated group included six patients who responded to treatment (○), one patient who had a transient response (*), and one patient who had no response (■). The other two groups included patients with infection with hepatitis C virus (○), infection with hepatitis B virus and positive (▲) or negative (●) status for viral DNA in serum, primary biliary cirrhosis (□), and cirrhosis associated with ulcerative colitis (Δ).

The mean value for TGFβ1 expression in the group of patients with high fibrogenic activity who were not treated with interferon was significantly higher than the mean in the other three groups shown ($P < 0.001$).

liver specimens. H3 mRNA was detectable by Northern blot analysis of specimens (Fig. 5) in all 22 patients with cirrhosis, but in only 11 of 20 patients (55 percent) with chronic hepatitis ($P < 0.01$ by Fisher's exact test). H3 mRNA was also detected in 8 of the 12 control patients, although in very low amounts (Fig. 5).

The pattern of expression of TGFα mRNA was similar to that of H3 histone (Fig. 5). TGFα mRNA was detectable in all patients with cirrhosis and in 60 percent of those with chronic hepatitis ($P < 0.01$ by Fisher's exact test) and was present in very low

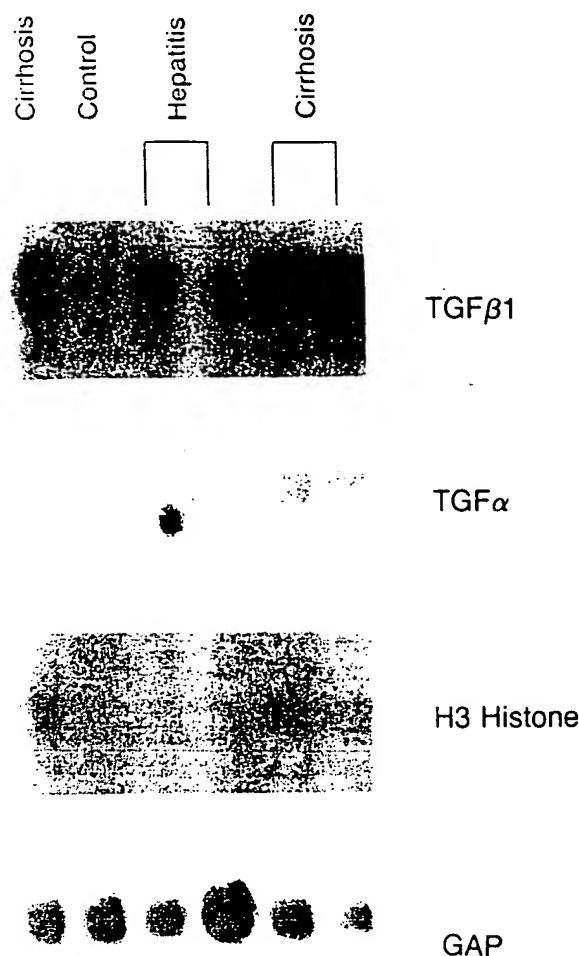


Figure 5. Representative Northern Blot Analysis of TGF β 1, TGF α , and H3 Histone mRNAs in Liver Specimens.

TGF α and H3 mRNAs were detected in patients with cirrhosis but were barely detectable in patients with chronic hepatitis or control patients. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAP) mRNA is shown for comparison.

amounts in 4 of the 12 control patients. However, the intensity of the TGF α bands in the Northern blots was lower than that of the H3 histone mRNA (Fig. 5) and close to the limit of sensitivity of the method. Thus, although the determinations of H3 histone mRNA in all patients could be used for quantitative analysis, quantitation of TGF α mRNA was possible in only 16 patients. In these samples the expression of H3 histone mRNA correlated with that of TGF α mRNA ($r = 0.70$, $P < 0.01$; data not shown).

Effect of Interferon Treatment

TGF β 1 mRNA Expression

Eight patients with chronic hepatitis C virus infection (cirrhosis or chronic hepatitis) and high fibrogenic activity were treated for one year with interferon as described in Methods. Six of these patients (Fig. 4) had a sustained clinical response to treatment,

with normalization of serum procollagen Type III peptide levels (concentration before vs. after therapy, 16.4 ± 4.0 vs. 9.3 ± 1.3 μ g per liter; $P < 0.05$ by Wilcoxon's signed-rank test) and serum ALT activity. In all six of these patients, hepatic TGF β 1 mRNA expression was in the normal range at the end of the treatment period and did not differ from expression in untreated patients with normal fibrogenic activity or the control patients. TGF β 1 mRNA expression was normal in one patient who had only a transient response to interferon (indicated by the asterisk in Fig. 4), but it remained high in the patient who did not respond to the treatment (indicated by the square in Fig. 4; the serum level of Type III peptide in this patient was 16.0 μ g per liter after therapy). Representative Northern blot analyses of TGF β 1 mRNA in liver specimens of interferon-treated and untreated patients are shown in Figure 6. The expression of the mRNA was much lower in the treated and the control patients than in the untreated patients, with the exception of one patient with chronic hepatitis and normal fibrogenic activity, who had low levels of TGF β 1 mRNA (third lane from left, Fig. 6).

H3 Histone mRNA Expression and Tissue Injury

The expression of H3 histone mRNA in the patients treated with interferon was similar to that in the control patients (Fig. 7). This was true not only of the six patients who responded to interferon but also of the two who had a transient response or no response to treatment (indicated by the asterisk and square in Fig.

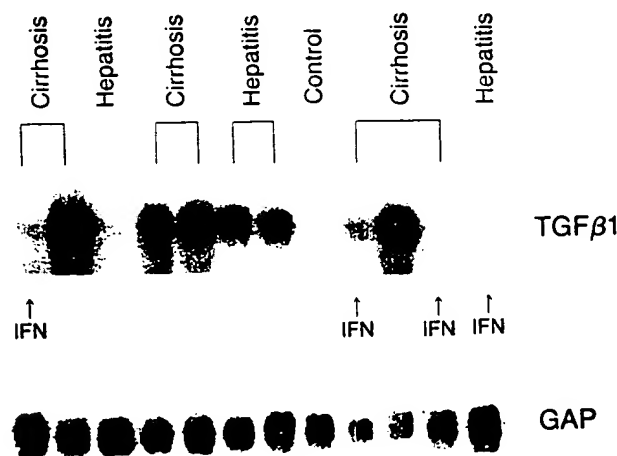


Figure 6. Representative Northern Blot Analysis of TGF β 1 mRNA in Liver Specimens from Patients with Chronic Liver Disease Untreated or Treated with Interferon.

Blots are shown for a control patient, patients with cirrhosis, patients with chronic hepatitis, and patients infected with hepatitis C virus treated with interferon (IFN) for one year. Note that in the treated patients, TGF β 1 mRNA expression was much lower than in untreated patients, with the exception of one untreated patient with chronic hepatitis and normal fibrogenic activity (third lane from left; a member of the group with normal fibrogenic activity shown in Fig. 4). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAP) mRNA is shown for comparison.

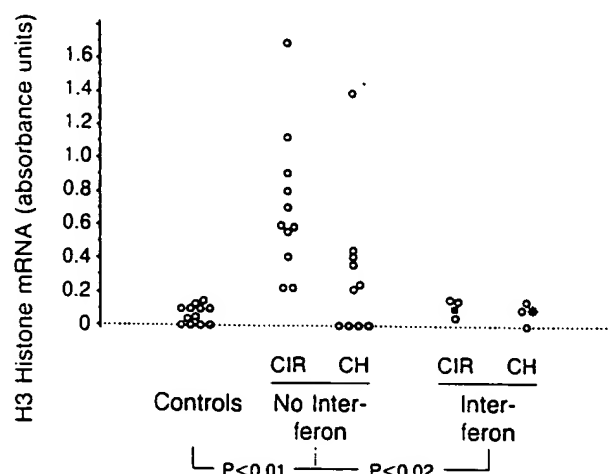


Figure 7. Effect of Interferon Therapy on Hepatic H3 Histone mRNA Expression in Patients with Chronic Hepatitis C Virus Infection.

The H3 mRNA content of each specimen was normalized to that of glyceraldehyde-3-phosphate dehydrogenase mRNA, as described in Methods.

The symbols represent the same patients shown in Figure 4. CIR denotes cirrhosis, and CH chronic hepatitis.

7). Portal and lobular inflammation and the degree of histologic damage decreased (Table 1) in the seven patients who had some response to treatment (six patients with a sustained response and one with a transient response).

DISCUSSION

Although the changes in liver architecture that occur in cirrhosis have been described in detail, the pathogenesis of fibrosis in this process is largely unknown. Collagens Type I and III, present in approximately equal amounts, constitute about 80 percent of the total collagen content of the normal human liver.^{1,4,7} In fibrosis, the amounts of collagen increase, but the increases in Type I collagen are generally greater than those in Type III, increasing the ratio of Type I to Type III twofold to fourfold.^{1,4,5} It is

not known whether these changes are due to increased collagen synthesis, decreased collagen degradation, or both.

The expression of procollagen Type I mRNA in the liver is a good indicator of fibrogenic activity.^{7,10} The serum level of procollagen Type III peptide also serves as a marker for liver fibrogenesis, although some peptide may be derived from collagen degradation.^{1,4,7,41} We found that the expression of TGFβ1 mRNA in the liver correlated directly with both the hepatic level of procollagen Type I mRNA and the serum level of collagen Type III peptide. In patients with increased fibrogenic activity (indicated by high serum levels of procollagen Type III peptide), the expression of hepatic TGFβ1 mRNA was 2 to 14 times higher than that in the control patients. These results indicate that collagen synthesis is an important component of fibrogenesis in chronic liver disease, and are in agreement with the work of Annoni et al.⁴² Furthermore, the results suggest that TGFβ1 may play a part in the pathogenesis of fibrosis in chronic hepatitis and cirrhosis.

In cultured cells, TGFβ1 stimulates the synthesis of collagens and other extracellular matrix components and retards their degradation.^{16,20} In addition, TGFβ1 is capable of activating lipocytes, cells that are probably a major site of synthesis of matrix proteins in chronic liver disease.^{1,4,6,7,21-24,43-49} A parallel increase in hepatic TGFβ1 and procollagen mRNAs has been found in experimental models of liver fibrosis.^{21,24} In particular, Nakatsukasa et al.²⁴ found increased levels of TGFβ1 mRNA in lipocytes, myofibroblasts, and fibroblasts in rats with carbon tetrachloride-induced liver fibrosis. In the early stages of fibrosis, TGFβ1 mRNA was also detected in inflammatory cells infiltrating the liver. Similar studies are necessary to determine which cells may be the sources of TGFβ1 mRNA in the livers of patients with chronic hepatitis or cirrhosis. Nevertheless, it is reasonable to speculate that in the development of cirrhosis, TGFβ1 produced by lipocytes and perhaps other nonparenchymal cells induces collagen synthesis in these same cells.

A substantial proportion of patients with chronic hepatitis C have improved during treatment with interferon alfa.⁵⁰⁻⁵³ Although the response to treatment was sometimes transient, the lobular and periportal inflammation of the liver regressed in many patients. As part of a larger study of the effects of this form of interferon in patients with chronic hepatitis C,³² we determined the amount of TGFβ1 mRNA and procollagen Type I mRNA in liver specimens from patients treated with interferon for one year. Patients who responded to treatment had normalization of the biochemical indicators of fibrogenesis (serum level of procollagen Type III peptide and expression of Type I procollagen mRNA in the liver) and normal amounts of liver TGFβ1 mRNA at the end of treatment. Although the number of patients studied was small, the results suggest that treatment with inter-

Table 1. Changes in the Histologic Activity Index and Subindexes in Patients with Chronic Hepatitis C Virus Infection Who Responded to Therapy with Interferon.*

INDEX	BEFORE THERAPY	AFTER THERAPY
Histologic activity index	12.4±3.2	5.7±2.0†
Subindexes		
Portal inflammation	3.7±0.5	2.6±0.8†
Piecemeal necrosis	4.1±1.6	0.85±1.2†
Lobular injury	2.6±1.1	0.71±0.5†
Fibrosis	2.3±0.9	1.6±1.1

*Values are means ±SD in seven of eight patients with chronic hepatitis C virus infection; the scores did not change in the patient who did not respond to treatment. Indexes were calculated according to the method of Knodell et al.²⁹

†P<0.05 by Wilcoxon's signed-rank test.

feron decreases fibrogenesis. In this and other studies,^{20,22,53} the histopathological score for liver fibrosis changed little during treatment although the scores for portal inflammation, piecemeal necrosis, and lobular injury decreased. Perhaps the degradation and resorption of fibrous tissue requires more than 12 months, as it does in patients with cirrhosis treated with colchicine.⁵⁴ We do not know whether the effect of interferon on Type I procollagen mRNA is a consequence of its action on TGF β 1 expression or whether the effect depends on the inhibition of viral replication. In experimental models of liver fibrosis that did not involve viral infection, interferon gamma decreased the expression of procollagen I and III mRNAs in liver cells in a dose-dependent manner, both in vivo and in vitro.⁵⁵

TGF α mRNA was detected in the livers of all patients with regenerative nodules, but only in some patients with chronic hepatitis but no cirrhosis. In addition, the expression of TGF α mRNA closely correlated with that of H3 histone mRNA, a good indicator of cell proliferation.²⁸⁻³⁰ These findings suggest that in patients with cirrhosis, TGF α is associated with hepatic-cell proliferation as in liver regeneration in rats after partial hepatectomy.³¹ Hepatocellular necrosis is an important initiating event in both extracellular matrix deposition and cell proliferation (regeneration) in cirrhosis.^{2,4,9,27} In patients with chronic hepatitis C who had a sustained response to interferon treatment, serum ALT activity and H3 histone mRNA expression were normal.

In summary, TGF β 1 may have an important role in fibrogenesis in chronic hepatitis and cirrhosis, and interferon therapy in patients with chronic hepatitis C can decrease the expression of TGF β 1 mRNA and procollagen Type I mRNA in the liver. Furthermore, we suggest that TGF α may be associated with cell proliferation in patients with chronic liver disease.

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